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The melanoma differentiation associated gene *mda*-7 suppresses cancer cell growth

(subtraction hybridization/DNA transfection/tumor suppressor/antisense expressing recombinant adenovirus/peptide antibody)

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ABSTRACT **Cancer is a disease characterized by defects in growth control, and tumor cells often display abnormal patterns of cellular differentiation. The combination of recombinant human fibroblast interferon and the antileukemic agent mezerein corrects these abnormalities in cultured human melanoma cells resulting in irreversible growth arrest and terminal differentiation. Subtraction hybridization identifies a melanoma differentiation associated gene (*mda*-7) with elevated expression in growth arrested and terminally differentiated human melanoma cells. Colony formation decreases when *mda*-7 is transfected into human tumor cells of diverse origin and with multiple genetic defects. In contrast, the effects of *mda*-7 on growth and colony formation in transient transfection assays with normal cells, including human mammary epithelial, human skin fibroblast, and rat embryo fibroblast, is quantitatively less than that found with cancer cells. Tumor cells expressing elevated *mda*-7 display suppression in monolayer growth and anchorage independence. Infection with a recombinant type 5 adenovirus expressing antisense *mda*-7 eliminates *mda*-7 suppression of the *in vitro* growth and transformed phenotype. The ability of *mda*-7 to suppress growth in cancer cells not expressing or containing defects in both the retinoblastoma (RB) and p53 genes indicates a lack of involvement of these critical tumor suppressor elements in mediating *mda*-7-induced growth inhibition. The lack of protein homology of *mda*-7 with previously described growth suppressing genes and the differential effect of this gene on normal versus cancer cells suggests that *mda*-7 may represent a new class of cancer growth suppressing genes with antitumor activity.**

Cancer is a complex multifactor and multistep process involving the coordinated expression and suppression of genes functioning as positive and negative regulators of oncogenesis (1–5). Direct cloning strategies, based on transfer of a dominant transforming or tumorigenic phenotype, have identified positive acting oncogenes (6–9). In contrast, the detection and cloning of genes that suppress the cancer phenotype have proven more difficult and elusive (10–15). A direct approach for isolating genes directly involved in regulating growth and differentiation involves subtraction hybridization between cDNA libraries constructed from actively growing cancer cells and cDNA libraries from cancer cells induced to lose proliferative capacity irreversibly and terminally differentiate (13, 14). This experimental strategy has been applied to human melanoma cells, induced to terminally differentiate by treatment with recombinant human interferon β (IFN-β) and mezerein (MEZ), resulting in the cloning of novel melanoma differentiation-associated (*mda*) genes not previously described in DNA data bases (13, 14). A direct role for specific *mda* genes in mediating growth and cell cycle control is apparent by the identification and cloning of *mda*-6 (13–16), which is identical to the ubiquitous inhibitor of cyclin-dependent kinases p21 (17). The importance of p21 in growth control is well documented, and this gene has been independently isolated, as WAF-1, CIP-1, and SDI-1, by a number of laboratories using different approaches (18–20). These studies indicate that specific genes associated with proliferative control are induced and may contribute to the processes of growth arrest and terminal differentiation in human cancer cells.

The *mda*-7 gene was cloned from a differentiation inducer (IFN-β plus MEZ)-treated human melanoma (H0-1) subtracted library (13, 14). The full-length *mda*-7 cDNA is 1718 nt, and the major open reading frame encodes a novel protein of 206 aa with an M_r of 23,800 (21). Previous studies indicate that *mda*-7 is induced as a function of growth arrest and induction of terminal differentiation in human melanoma cells (14, 21). *mda*-7 expression also inversely correlates with melanoma progression—i.e., actively growing normal human melanocytes express more *mda*-7 than metastatic human melanoma cells (21). Moreover, *mda*-7 is growth inhibitory toward human melanoma cells in transient transfection assays and in stable transformed cells containing a dexamethasone (DEX)-inducible *mda*-7 gene (21). These studies indicate that *mda*-7 may contribute to the physiology of human melanocytes and melanomas, and this gene has growth suppressive properties when overexpressed in human melanoma cells.

We report that *mda*-7 is a potent growth suppressing gene in cancer cells of diverse origin, including breast, central nervous system, cervix, colon, prostate, and connective tissue. An inhibition in colony formation occurs in cancer cells containing defects in their p53 and/or retinoblastoma (RB) genes or lacking p53 and RB expression. In contrast, expression of *mda*-7 in normal human mammary epithelial cells, human skin fibroblasts and rat embryo fibroblasts induces quantitatively less growth suppression than in cancer cells. When stably expressed in human cervical carcinoma (HeLa) and prostate carcinoma (DU-145) cells, *mda*-7 has a negative effect on growth and transformation-related properties. The effects of *mda*-7 on HeLa cells are reversible following abrogation of the MDA-7 protein by infection with a genetically modified Ad5 vector expressing an antisense *mda*-7 gene. These observations indicate that *mda*-7 is a novel growth

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Abbreviations: IFN-β, interferon β; MEZ, mezerein; *mda*, melanoma differentiation-associated; Hyg^R, hygromycin resistant; DEX, dexamethasone; Ad5, type 5 adenovirus; RB, retinoblastoma; HMEC, human mammary epithelial cell; (S), sense; (AS), antisense; Ad, adenovirus; HMC, high molecular weight complexing protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RSV, Rous sarcoma virus.

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suppressing gene with a wide range of inhibitory actions in human cancers manifesting different genetic defects.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Human carcinoma cell lines, including MCF-7 and T47D (breast), LS174T and SW480 (colorectal), HeLa (cervical), DU-145 (prostate), and HONE-1 (nasopharyngeal) (9, 22–25), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (DMEM-10) at 37°C in a 5% $C0_2$/95% air-humidified incubator. Additional human cell types including HBL-100 (normal mammary epithelial), H0-1 and C8161 (melanoma), GBM-18 and T98G (glioblastoma multiforme), and Saos-2 (human osteosarcoma) were maintained under similar conditions. Early passage normal human mammary epithelial cells (HMEC; passages 10–12) were obtained from Clonetics (San Diego). HMECs were maintained in serum-free medium as described by Clonetics. CREF-Trans 6 (cloned Fischer rat embryo fibroblast) (9, 26) and CREF-Ha-*ras* (CREF cells transformed by the Ha-*ras* (T24) oncogene) (27) were cultured in DMEM-5. HeLa cl 1 is a hygromycin resistant (Hyg^R) Rous sarcoma virus (RSV) vector (pREP4) (Invitrogen) transformed HeLa clone. HeLa cl 2 is a Hyg^R *mda*-7 expressing HeLa clone. HeLa cl 1 and HeLa cl 2 cells were constructed as described (12, 21) and maintained in DMEM-10 containing 100 μg of hygromycin of ml. DU-145 cl 6 and DU-145 cl 7 cells contain a DEX-inducible *mda*-7 gene (cloned in a pMAMneo vector) (Clontech) (21) and are maintained in DMEM-10 containing 200 μg of G418 per ml.

Subtraction Hybridization, Plasmids, Expression Vector Constructs, and Northern Hybridization. Identification and cloning of *mda*-7 by subtraction hybridization was achieved as described (13). A full-length *mda*-7 cDNA was isolated by screening a recombinant IFN-β plus MEZ-treated H0-1 cDNA library (13) and using the procedure of rapid amplification of cDNA ends as described (15). An *mda*-7 cDNA fragment (nucleotide position 176–960) containing the open reading frame was amplified with PCR and cloned into pCRII (Invitrogen). The orientation of the inserts in the vectors was determined by restriction mapping. The human cell expression constructs were made by cloning *Kpn*I–*Xho*I fragments from the PCR™ vectors into pREP4 vector (Invitrogen) downstream of a RSV promoter in a sense [*mda*-7 (S)] or antisense [*mda*-7 (AS)] orientation. Alternatively, the *mda*-7 gene fragment was cloned into the pMAMneo (Clontech) vector in a sense and antisense orientation. RNA isolation and Northern blotting were performed as described (9, 12, 13, 21).

Monolayer Growth, Anchorage-Independence, and DNA-Transfection Assays. Monolayer and anchorage-independent growth assays were performed as previously described (8, 12, 26). To study the effect of *mda*-7 on monolayer colony formation the vector [pREP4 (RSV)] containing no insert, *mda*-7 (S) or *mda*-7 (AS) expression constructs were transfected into the various cell types by the lipofectin method (GIBCO/BRL) and Hyg^R colony formation or cell growth in hygromycin was determined (12, 21).

Construction of *mda*-7 (AS) Adenovirus (Ad) Vector. The recombinant replication-defective Ad.*mda*-7 (AS) was created in two steps. First, the coding sequence of the *mda*-7 gene was cloned into a modified Ad expression vector pAd.CMV (28). This contains, in order, the first 355 bp from the left end of the Ad genome, the cytomegalovirus immediate early promoter, DNA encoding splice donor and acceptor sites, cloning sites for the desired gene (in this case *mda*-7), DNA encoding a polyA signal sequence from the β globin gene, and ≈3 kbp of adenovirus sequence extending from within the E1B coding region. This arrangement allows high level expression of the cloned sequence by the cytomegalovirus immediate early gene promoter, and appropriate RNA processing (28). The recombinant virus was created *in vivo* in 293 cells (29) by homologous recombination between *mda*-7-containing vector and plasmid JM17, which contains the whole of the Ad genome cloned into a modified version of pBR322 (30). JM17 gives rise to Ad genomes *in vivo* but they are too large to package. This constraint is relieved by recombination with the vector to create a packageable genome (30), containing the gene of choice. The recombinant virus is replication defective in human cells except 293 cells, which express adenovirus E1A and E1B. Following transfection of the two plasmids, infectious virus was recovered, the genomes were analyzed to confirm the recombinant structure, and then virus was plaque purified, all by standard procedures (31).

Peptide Antibody Production and Immunoprecipitation Analyses. Peptide antibodies were prepared against PSQEN-EMFSIRD as described (21). Logarithmically growing HeLa, HeLa cl 1 (Hyg^R pREP4 vector control HeLa clone), and HeLa cl 2 [pREP4-*mda*-7 (S) transfected Hyg^R *mda*-7 expressing HeLa clone] cells were either untreated or infected with 10 plaque forming units of control adenovirus (H5*dl*434) (32) or a recombinant adenovirus expressing *mda*-7 (AS) [Ad.*mda*-7 (AS)]. At various times after infection, cultures were starved of methionine for 1 hr at 37°C in methionine-free medium, and cells were concentrated by pelleting and labeled for 4 hr at 37°C in 1 ml of the same medium with 100 μCi (1 Ci = 37 GBq) of ^{35}S (NEN; Express ^{35}S). Immunoprecipitation analyses with 2 μg of *MDA*-7 peptide rabbit polyclonal antibody or actin monoclonal antibody (Oncogene Sciences) were performed as described (15, 21).

RESULTS

Enhanced Growth Inhibitory Properties of *mda*-7 in Human Cancer Cells and Ha-*ras*-Transformed Rat Embryo Fibroblast Cells. DNA transfection assays were performed to evaluate the effect of elevated expression of *mda*-7 on cell growth. When transfected into human cervical carcinoma (HeLa) cells, the *mda*-7 (S) construct results in a 10- to 15-fold reduction in Hyg^R colonies in comparison with the pREP4 vector and *mda*-7 (AS) construct transfected cultures (Fig. 1 and Table 1). In addition to forming fewer colonies, *mda*-7 (S) colonies are generally smaller in size than corresponding Hyg^R colonies resulting after transfection with the pREP4 vector or *mda*-7 (AS) constructs (Fig. 1). When transfected into additional human cancer cell lines, *mda*-7 (S) constructs reduce Hyg^R colony formation by 3- to 10-fold (Table 1 and data not shown). These include human breast carcinoma (MCF-7 and T47D), colon carcinoma (LS174T and SW480), nasopharyngeal carcinoma (HONE-1), prostate carcinoma (DU-145), melanoma (H0-1 and C8161), glioblastoma multiforme (GBM-18 and T98G), and osteosarcoma (Saos-2). As observed with HeLa cells, the average sizes of Hyg^R colonies that form after transfection with *mda*-7 (S) constructs are smaller than those formed following transfection with the empty pREP4 vector or *mda*-7 (AS) constructs. These results demonstrate that *mda*-7 is a potent growth suppressing gene when overexpressed in a wide spectrum of histologically distinct human cancers.

To determine if *mda*-7 also inhibits the growth of normal cells and whether this effect is quantitatively similar to that observed with human cancer cells, transient DNA transfection assays were performed with passage 10 to 12 normal HMECs, the normal breast epithelial cell line HBL-100, normal human skin fibroblasts (passage 21) and a cloned normal rat embryo fibroblast cell line (CREF-Trans 6) (7, 8). Since HMEC, HBL-100, and normal human skin fibroblasts do not form well-defined colonies at high frequencies, even when using a feeder-layer, the effect on total cell number after transfection with the different RSV constructs and growth for 2 and 3 weeks in hygromycin was determined. Using this approach, an ≈1.1- to 1.6-fold decrease in HMEC, an ≈1.1- to 1.2-fold

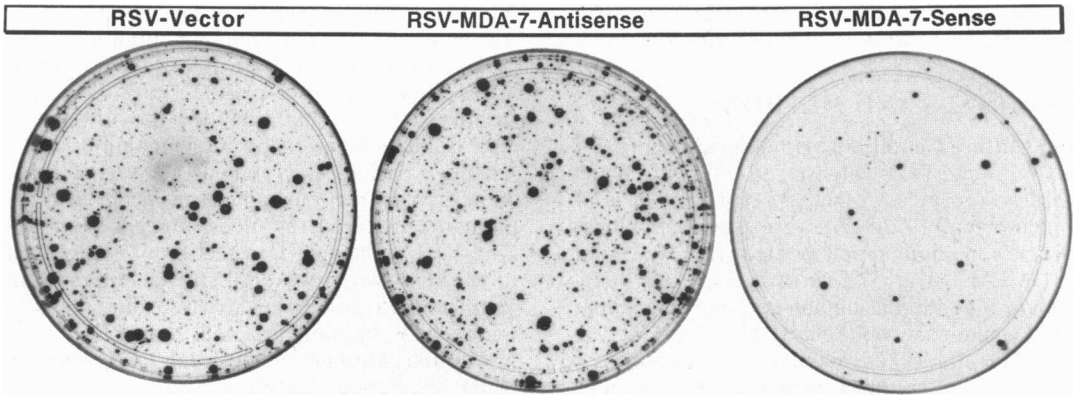

FIG. 1. Effect of *mda*-7 expression on Hyg^R colony formation in HeLa cells. HeLa cells were transfected with 10 μg of pREP4 vector (RSV-vector), *mda*-7 cloned in an antisense orientation in the pREP4 vector (RSV-MDA-7-Antisense), or *mda*-7 cloned in a sense orientation in the pREP4 vector (RSV-MDA-7-Sense) and selected in media containing 100 μg of hygromycin.

decrease in HBL-100 and an ≈1.3- to 2.1-fold decrease in normal human skin fibroblast cell number was observed (three independent experiments with each cell type) in *mda*-7 (S) versus *mda*-7 (AS) or pREP4 vector transfected normal cells, respectively (data not shown). In contrast, using a similar experimental protocol with T47D human breast carcinoma cells, growth was inhibited following transfection with the *mda*-7 (S) construct ~3.2 to 5.2-fold in comparison with vector- and antisense-transfected cells (data not shown). In the case of CREF-Trans 6 cells, the difference in Hyg^R colony formation for six independent transfection assays between *mda*-7 (S) versus *mda*-7 (AS) and vector transfected cells ranged from 0.5- to 2.8-fold (Table 1 and data not shown). In contrast, transfection of *mda*-7 (S) constructs into Ha-*ras*-transformed CREF cells reduced colony formation by ≈6- to 8-fold (Table 1 and data not shown). These results indicate that *mda*-7 is quantitatively less effective in reducing growth and colony formation in normal human and normal rodent cells than in human cancer and Ha-*ras*-transformed rat embryo cells.

Effect of Stable and Inducible *mda*-7 Expression and Antisense Inhibition of *mda*-7 Expression on Cell Growth and the Transformed Phenotype. To determine the reason for low frequency HeLa cell survival after transfection with the *mda*-7 (S) gene, 10 independent Hyg^R colonies were isolated following transfection with the *mda*-7 (S) construct. Of the 10 clones analyzed by Northern blotting for *mda*-7 expression, 7 clones did not express detectable *mda*-7 mRNA, 2 clones expressed low levels of *mda*-7 mRNA, and 1 clone (designated HeLa cl 2) displayed high levels of *mda*-7 mRNA (data not shown). In contrast, all of the clones displayed comparable levels of Hyg^R and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression (data not shown). When compared with parental HeLa cells or an pREP4 vector HeLa clone (designated HeLa cl 1), HeLa cl 2 (*mda*-7 expressing) cells grew at a reduced rate (Fig. 2). When grown in agar, uncloned HeLa and HeLa cl 1 cells grew with ≈42% efficiency, whereas HeLa cl 2 (*mda*-7 expressing) cells grew with ≈25% efficiency and the average sizes of colonies were smaller than observed with parental HeLa and pREP4 vector HeLa cl 1 cells. These results indicate that HeLa survival after transfection with *mda*-7 results primarily from the lack of or low levels of *mda*-7 expression. However, in HeLa cells that stably express elevated *mda*-7, growth in monolayer culture and anchorage-independence are reduced.

To determine if the reduction in *in vitro* growth and transformation suppression found in HeLa cl 2 (*mda*-7 expressing) are a direct consequence of *mda*-7 expression, we used an antisense strategy to directly inhibit *mda*-7 expression. A recombinant Ad5 vector containing the *mda*-7 gene cloned in an antisense orientation [Ad.*mda*-7 (AS)] was constructed. Infection of HeLa cl 2 (*mda*-7 expressing), but not HeLa cl 1 (pREP4 vector, non-*mda*-7 expressing) or parental HeLa, with Ad.*mda*-7 (AS) increases growth rate and agar cloning efficiency (from ≈25 to ≈44%) (Fig. 2 and data not shown). In contrast, the

Table 1. Effect of *mda*-7 on monolayer colony formation of human cancer, normal rat embryo fibroblast (CREF) and Ha-ras-transformed CREF cells

Cell Type	RSV-vector*	RSV-*mda*-7 (S)†		RSV-*mda*-7 (AS)
Human cancer cell lines‡				
MCF-7 (breast-Ca)	118 ± 24	42 ± 16	(3.5)	146 ± 20
T47D (breast-Ca)	172 ± 9	44 ± 7	(4.2)	186 ± 28
HeLa (cervix-Ca)	1571 ± 446	117 ± 107	(15.2)	1771 ± 385
LS174T (colorectal-Ca)	130 ± 14	30 ± 3	(5.4)	160 ± 15
HONE-1 (nasopharyngeal-Ca)	219 ± 19	71 ± 8	(3.5)	250 ± 19
DU-145 (prostate-Ca)	174 ± 18	54 ± 8	(3.1)	166 ± 12
T98G (glioblastoma)	99 ± 9	32 ± 4	(3.6)	115 ± 14
Saos-2 (osteosarcoma)	126 ± 22	35 ± 6	(3.9)	138 ± 14
Rat embryo fibroblast				
CREF (normal rat embryo)	60 ± 10	35 ± 5	(1.7)	66 ± 7
CREF-*ras* (transformed)	147 ± 16	25 ± 4	(6.0)	151 ± 16

*Logarithmically growing cells were seeded at 1 × 10^6 cells per 100-mm plate and transfected with 10 μg of vector [pREP4 (RSV)] containing no insert, *mda*-7 (S), or *mda*-7 (AS). After 24 hr, cells were replated at ≈2 × 10^5 cells per 100-mm plate in medium containing 100 μg of hygromycin per ml. Medium was changed every 3 or 4 days and plates were fixed in formaldehyde and stained with Giemsa at day 14 or 21. Colonies containing 50 or more cells were enumerated. Values shown are the average Hyg^R colonies formed in four to five replicate plates ± SD.

†Values in parentheses indicate fold-decrease in colony formation versus RSV-*mda*-7 (AS) transfected cells.

‡MCF-7, T47D, HeLa, LS174T, DU-145, and HONE-1 are human carcinoma (Ca) cell lines isolated from the indicated anatomical site. T98G is a human glioblastoma multiforme cell line. CREF-*ras* is a Ha-*ras* (T24) oncogene transformed CREF clone.

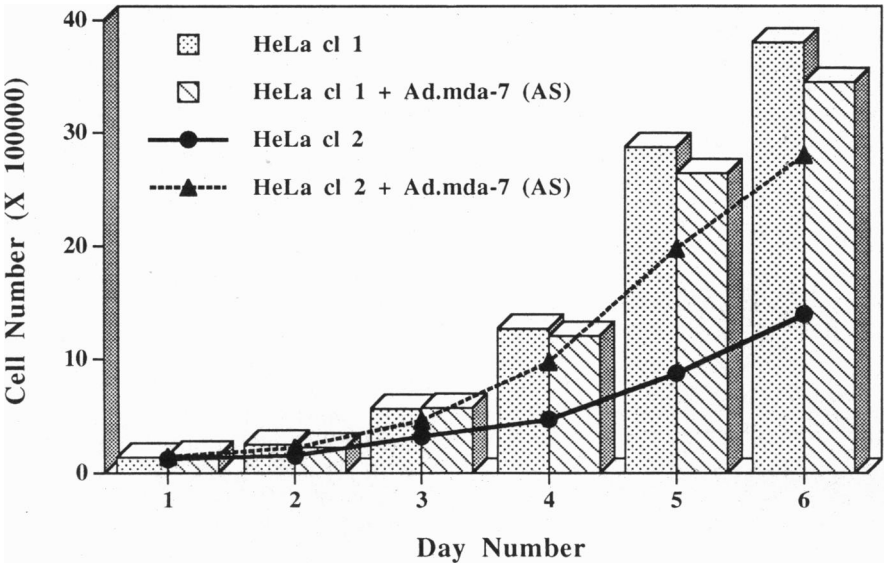

FIG. 2. Effect of antisense *mda*-7 on monolayer growth of pREP4 vector HeLa cl 1 and *mda*-7 (S) expressing HeLa cl 2 cells. HeLa cl 1 (pREP4 vector transformed HeLa clone) and HeLa cl 2 (*mda*-7 expressing HeLa clone) cells were grown in the absence or following infection with 10 plaque forming units/cell with a recombinant type 5 adenovirus (Ad5) expressing antisense *mda*-7 [Ad.*mda*-7 (AS)]. Results are the average cell number from triplicate samples that varied by ≤10%.

control mutant Ad5 vector (H5*dl*434), not containing the *mda*-7 gene, does not affect monolayer or agar growth of parental HeLa, HeLa cl 1 or HeLa cl 2 cells (data not shown).

Using *mda*-7-specific peptide antibodies produced in rabbits and immunoprecipitation analyses, we demonstrate that HeLa cl 2 (*mda*-7 expressing) cells contain elevated levels of the MDA-7 ≈24-kDa protein and a high molecular weight complexing (HMC) protein of ≈90 to 110 kDa (Fig. 3). Infection with Ad.*mda*-7 (AS), but not the H5*dl*434 control non-*mda*-7 expressing virus, results in a temporal decrease in both the ≈24-kDa MDA-7 protein and the HMC protein (21) (Fig. 3). Reduced levels of both proteins are seen by 48 hr and remain suppressed over a 96-hr period after infection with Ad.*mda*-7 (AS). In contrast, actin levels remain unaltered following viral infection. These findings indicate that antisense inhibition of *MDA*-7 protein expression in HeLa cl 2 (*mda*-7 expressing) can directly extinguish *mda*-7-induced growth suppression and inhibition in anchorage-independent growth.

To confirm the suppressive effect of *mda*-7 on cell growth, DU-145 human prostate cancer cells were engineered to express a DEX-inducible *mda*-7 gene. When DU-145 cl 6 or cl 7 cells [containing a DEX-inducible *mda*-7 (S) gene], but not parental DU-145 cells, are grown for 24 to 96 hr in the presence of 10^{-6} M DEX, *mda*-7 mRNA and protein (including the HMC protein) are induced (Fig. 4 and data not shown). In contrast, DEX does not alter neomycin resistance (Neo^R) gene expression in DU-145 cl 6 and cl 7 cells or GAPDH expression in any of the cells tested (Fig. 4). Induction of *mda*-7 expression in DU-145 cl 6 and cl 7 cells by growth in 10^{-6} M DEX results in ≈50% reduction in cell number after 96 hr versus growth in the absence of DEX (data not shown). In contrast, no significant growth inhibition occurs when parental DU-145 or pMAMneo vector transformed DU-145 cells are grown for 96 hr in medium containing 10^{-6} M DEX (data not shown). These data indicate that ectopic expression of *mda*-7 can directly alter cell growth in prostate cancer cells.

DISCUSSION

Subtraction hybridization identified *mda* genes with elevated expression in growth arrested and terminally differentiated human melanoma cells (13, 14, 21). Determining the function

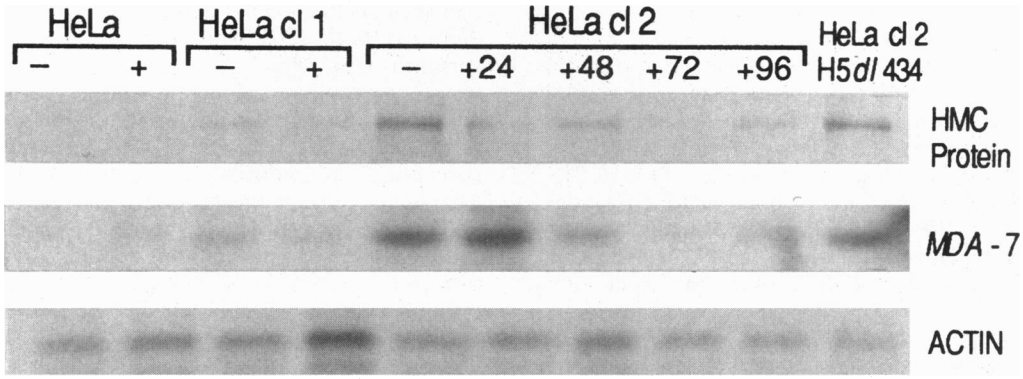

FIG. 3. Effect of antisense *mda*-7 on the high molecular weight-*MDA*-7 complexing (HMC) protein, the *MDA*-7 protein, and the actin protein in HeLa, HeLa cl 1, and HeLa cl 2 cells. HeLa and HeLa cl 1 (pREP4 vector transformed HeLa clone) were uninfected (−) or infected (+) with 10 plaque forming units/cell of Ad.*mda*-7 (AS) for 96 hr and labeled with [^{35}S]methionine, and the levels of the HMC, MDA-7, and actin proteins were determined by immunoprecipitation analysis. For HeLa cl 2 (*mda*-7 expressing HeLa clone), the effect of infection with 10 plaque forming units/ml of Ad.*mda*-7 (AS) on protein levels was determined by immunoprecipitation analysis of [^{35}S]methionine labeled cell lysates after +24, +48, +72, and +96 hr. The effect of infection of HeLa cl 2 cells with the control mutant Ad5, H5*dl* 434, was determined by immunoprecipitation analysis of [^{35}S]methionine labeled cell lysates 96 hr after infection with 10 plaque forming units/cell.

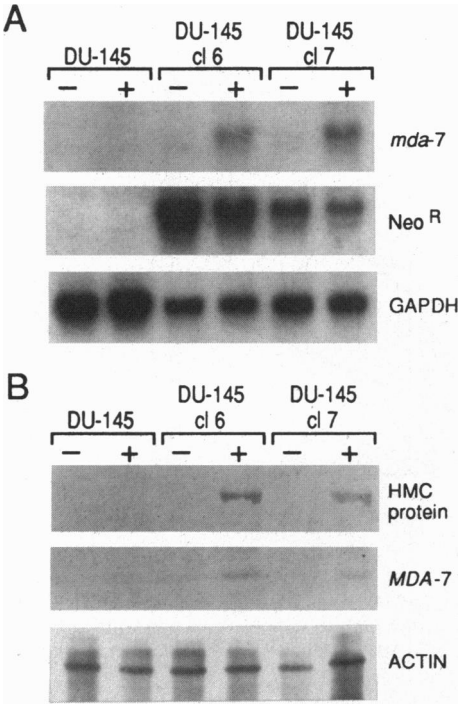

FIG. 4. Synthesis of *mda*-7 RNA and protein in DU-145 clones containing a DEX-inducible *mda*-7 gene. (*A*) Cells were grown in the absence or presence of 10^{-6} M DEX for 96 hr, and total RNA was isolated, subjected to Northern blotting, and probed with *mda*-7, a neomycin resistance (Neo^R) gene and GAPDH. (*B*) Cells were grown in the absence or presence of 10^{-6} M DEX for 96 hr, and cellular proteins were labeled with [^{35}S]methionine and immunoprecipitated with antibodies recognizing *MDA*-7 and actin proteins.

of these *mda* genes will be paramount in defining the molecular basis of growth control and terminal differentiation in human melanoma and other cell types. The *mda*-7 gene (14, 21) is now shown to be a ubiquitous growth suppressing gene when transiently or stably expressed in a wide array of human cancer cell lines. This finding extends our previous observation indicating growth inhibitory properties of the MDA-7 protein in human melanoma cells (21). In contrast to its effects on cancer cells, transfection of *mda*-7 into normal human mammary epithelial, normal human skin fibroblast and normal rat embryo fibroblast cells results in quantitatively less growth suppression. Like another *mda* gene, *mda*-6 (p21), *mda*-7 expression is also inversely correlated with melanoma progression, with elevated levels of both *mda*-6 (p21) and *mda*-7 present in normal human melanocytes relative to metastatic human melanoma cells (14–16, 21). Since normal melanocytes still retain proliferative capacity, although at a reduced rate relative to melanoma cells, it is possible that both *mda*-6 (p21) and *mda*-7 function as negative regulators of the progression phenotype in melanocyte/melanoma lineage cells (14–16, 21). Moreover, the elevated expression of both *mda*-6 (p21) and *mda*-7 in terminally differentiated and irreversibly growth arrested human melanoma cells, suggests that these genes may also be important regulators of the terminal differentiation phenotype (13–16, 21).

The mechanism by which *mda*-7 elicits its growth-suppressive effects on human cancer cells is not presently known. The structure of *mda*-7 does not provide insight into potential function, since no sequence motifs are present that would suggest a potential mode of action. The effect of *mda*-7 on cell growth can be distinguished from the extensively studied tumor suppressor gene p53 (33, 34). Transient expression of p53 in the mutant p53 containing T47D human breast carcinoma cell line results in growth suppression, whereas transfection of a wild-type p53 gene into the wild-type p53 containing MCF-7 human breast carcinoma cell line does not induce growth inhibition (34). In contrast, *mda*-7 induces similar growth suppression in both T47D and MCF-7 cells (Table 1). Growth inhibition by *mda*-7 can also be disassociated from that observed with the RB gene (pRB), the pRb-associated p107 gene, and the putative tumor suppressor gene $p16^{ink4}$ (25, 35). Overexpression of pRb and p107 inhibit cellular proliferation in specific cell types and in a cell cycle-dependent manner (35–37). Transfection of pRb or p107 into the human glioblastoma cell line T98G that contains an apparently normal RB gene (25) does not induce growth suppression (35, 37), whereas transient *mda*-7 (S) expression reduces T98G colony formation (Table 1). At the present time, the growth inhibitory effect of *mda*-7 cannot be distinguished from growth suppression induced by the RB family member p130/pRb2, which also inhibits proliferation in T98G cells (25). The $p16^{ink4}$ gene induces growth arrest in cells containing a functional RB gene (35, 37), whereas *mda*-7 growth suppression occurs in cells containing normal, abnormal, or nonfunctional RB genes. Transfection of *mda*-7 into the DU-145 human prostate carcinoma cell line that contains a mutated RB gene (38) and Saos-2 human osteosarcoma cells that do not express RB (or wild-type p53) results in an inhibition in colony formation (Table 1). Similarly, induction of *mda*-7 expression in stable DEX-inducible *mda*-7 transformed DU-145 clones results in growth suppression. These findings indicate a lack of dependence on a functional RB gene for growth inhibition by *mda*-7. Taken together these studies demonstrate that the inhibitory effect of *mda*-7 occurs by a mechanism that is distinct from the mode of action of the two most extensively studied tumor suppressor genes, p53 and pRb, and the putative tumor suppressor gene, $p16^{ink4}$.

Several genes have been identified that display elevated expression as a function of growth arrest or DNA damage in mammalian cells (39, 40). Three growth arrest and DNA damage inducible (*gadd*) genes, *gadd45*, *gadd153*, and *gadd34*, the closely related myeloid differentiation primary response (*MyD118*) gene (41), and the wild-type p53 inhibiting gene *mdm-2* (42) are upregulated in cells by treatment with the DNA damaging agent methyl methanesulfonate (40). The *gadd45* and growth arrest-specific gene (*gas1*) (43, 44) are induced by maintaining cells at confluence, serum-starving cells or growing cells in low serum (40, 43, 44). In contrast, *mda*-7 mRNA expression is not induced in human melanoma cells following treatment with methyl methanesulfonate or after maintaining cells at confluence (21). Moreover, only a small increase in *mda*-7 mRNA expression occurs in H0-1 human melanoma cells following growth in serum-free medium for 96 hr (21). The difference in regulation of *mda*-7 versus the *gadd*, *MyD118*, and *gas-1* genes indicates that *mda*-7 may represent a new class of growth arresting genes.

In summary, a negative growth regulator, *mda*-7, is described that induces growth suppression in human cancer cells containing both normal and mutated p53 and RB genes. Characterization of the genomic structure of *mda*-7 will be important in determining if this gene normally functions as a tumor suppressor gene and whether alterations are present in this gene in tumor versus normal cells. Identification of the promoter region of *mda*-7 will also permit an analysis of the mechanism by which this gene is differentially expressed and inducible by IFN-β plus MEZ in specific cell types. Of potential importance and warranting expanded studies is the finding that *mda*-7 is more growth inhibitory toward cancer and transformed cells than normal cells. In this context, *mda*-7 could prove useful as part of a gene-based interventional strategy for cancer therapy, in an analogous manner as the wild-type p53 gene is currently being tested for efficacy in the therapy of specific human malignancies.

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